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Deanna L. Funnell-Harris
USDA-ARS, Deanna.Funnell-Harris@ars.usda.gov

Patrick M. O'Neill
University of Nebraska-Lincoln, poneill4@unl.edu

Scott E. Sattler
USDA-ARS, Scott.Sattler@ars.usda.gov

Tammy Gries
University of Nebraska-Lincoln, tgries2@unl.edu

Mark A. Berhow
USDA-ARS, Mark.Berhow@ars.usda.gov

See next page for additional authors

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Response of sorghum stalk pathogens to *brown midrib* plants and soluble phenolic extracts from near isogenic lines

Deanna L. Funnell-Harris · Patrick M. O'Neill · Scott E. Sattler · Tammy Gries · Mark A. Berhow · Jeffrey F. Pedersen

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Abstract Sorghum [*Sorghum bicolor* (L.) Moench] has drawn attention as potential feedstock for lignocellulosic biofuels production, and reducing lignin is one way to increase conversion efficiency. Little research has been previously conducted to assess the response of reduced lignin sorghum lines to the Fusarium stalk rot pathogens *Fusarium verticillioides* and *Fusarium proliferatum* and the charcoal rot pathogen, *Macrophomina phaseolina*. Loss of function mutations in either the *Brown midrib* (*Bmr*) *6* or *12* gene that both encode a monolignol biosynthetic enzyme in the pathway that produces subunits of the lignin polymer, results in reduced lignin content. Near-isogenic *bmr6*, *bmr12,* and *bmr6 bmr12* lines had previously been developed, which were shown to have significantly reduced lignin content and increased levels of soluble phenolics. In the current study, these lines in two backgrounds were shown to not be more susceptible to *F. verticillioides*, *F. proliferatum* and *M. phaseolina* inoculations, and some *bmr* lines exhibited increased resistance to *F. proliferatum* and *M. phaseolina*, compared to wild-type lines. When the *Fusarium* stalk rot pathogen, *Fusarium thapsinum,* was grown on methanol soluble stalk extracts from *bmr6* and wild-type plants, it grew significantly faster on medium with *bmr6* extract than on wild-type extract or controls. This result suggested that factors other than soluble phenolics from the extract, such as cell wall bound phenolics or inducible defense compounds, contributed to increased resistance observed in *bmr6* plants.

Key words Fusarium stalk rot · Charcoal rot · *Sorghum bicolor* (L.) Moench · *brown midrib* · Phenolic metabolites · Bioenergy feedstocks

Abbreviations

Bmr6	Cinnamyl alcohol dehydrogenase biosynthetic gene
Bmr12	Caffeic acid *O*-methyltransferase biosynthetic gene
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PGA	Peptone glucose agar

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] biomass has traditionally been utilized for livestock feed, but has

Jeffrey F. Pedersen is retired.

D. L. Funnell-Harris (✉) · P. M. O'Neill
Wheat, Sorghum and Forage Research Unit (WSFRU), United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Department of Plant Pathology, University of Nebraska (UNL), Lincoln, NE 68583-0937, USA
e-mail: Deanna.Funnell-Harris@ars.usda.gov

S. E. Sattler · T. Gries · J. F. Pedersen
WSFRU, USDA-ARS, Department of Agronomy and Horticulture, UNL, Lincoln, NE, USA

M. A. Berhow
National Center for Agricultural Utilization Research, Crop Bioprotection Research Unit, USDA-ARS, 1815 N. University St, Peoria, IL 61604, USA

recently drawn attention as potential feedstock for lignocellulosic bioenergy production (Li et al. 2008; Sattler et al. 2010a). To convert biomass to ethanol, the sugar subunits must be hydrolyzed (saccaharification) from the polysaccharides cellulose and hemicellulose, two major components of plant cell walls, then fermented into ethanol (Sun and Cheng 2002). The third component of plant biomass, lignin, is always complexed with hemicellose through covalent bonds, thus impairing hydrolysis (Buranov and Mazza 2008). Therefore, biomass could be improved for bioenergy or livestock by reducing its lignin content or modifying its composition (Anderson and Akin 2008; Oliveira et al. 2015). However, lignin is also a critical factor for the success of terrestrial plants (Weng and Chapple 2010). It confers mechanical strength to stand upright and expand in size, it is a necessary component for long-distance water transport and it provides a natural barrier to pathogens and herbivores.

In order to address this potential trade-off between fitness and lowered lignin content, two mutant alleles, *brown midrib* (*bmr*)*-6* and *bmr12*, were incorporated into elite sorghum lines, resulting in several *bmr6* and *bmr12* lines near-isogenic to their wild-type counterparts (Pedersen et al. 2006; Pedersen et al. 2008). The bmr phenotype is associated with reduced lignin (Sattler et al. 2014). It was shown that *bmr6* or *bmr12* resulted in significantly reduced lignin content, increased digestibility for use as ruminant animal feed and increased conversion to lignocellulosic ethanol (Dien et al. 2009; Oliver et al. 2005). *Bmr6* encodes a cinnamyl alcohol dehydrogenase (Saballos et al. 2009; Sattler et al. 2009) and *Bmr12* encodes caffeic acid *O*-methyltransferase (Bout and Vermerris 2003). The *bmr6* and *bmr12* alleles used in the current study resulted from a nonsense mutation within their respective genes, and both are likely null alleles (Bout and Vermerris 2003; Palmer et al. 2008; Saballos et al. 2009; Sattler et al. 2009; Sattler et al. 2012; Scully et al. 2016). Near-isogenic *bmr6 bmr12* double mutant lines were also developed, which have significantly reduced lignin content as compared with either single *bmr* mutant line and increased ethanol conversion efficiency of the biomass over the single mutations (Dien et al. 2009; Pedersen et al. 2008). Further studies using *bmr6, bmr12* and *bmr6 bmr12* plants showed that concentrations of both soluble (μg g^{-1} dry weight) and cell wall-bound (μg g^{-1} cell walls) phenolic compounds were altered in the *bmr* lines, compared to their wild-type counterparts (Palmer et al. 2008), and *bmr6* plants had the highest concentrations of free phenolic compounds overall. However, interactions were apparent between genetic background and *bmr* that resulted in significant differences in the free phenolic profiles of the same mutation in different lines.

It was previously demonstrated that *bmr6* and *bmr12* were not more susceptible to fungal (e.g. no increased colonization) infection than wild-type, counter to previously-held concepts that lignin is an essential component of plant resistance (Funnell and Pedersen 2006; Funnell-Harris et al. 2010; Nicholson and Hammerschmidt 1992). Much of these studies focused on the Fusarium stalk rot pathogen, *Fusarium thapsinum* Klittich, J.F. Leslie, P.E. Nelson & Marasas 1997 (current name *Gibberella thapsina* Klittich, J.F. Leslie, P.E. Nelson & Marasas, to be referred to as *F. thapsinum*) (Funnell and Pedersen 2006; Funnell-Harris et al. 2014). In these studies, *bmr6, bmr12* and *bmr6 bmr12* double mutant lines were not more susceptible to basal stalk (second internode above the roots) inoculations and these lines could exhibit increased resistance (significantly shorter lesions) to peduncle (top of the stalk) inoculations. However, there are other virulent and prevalent stalk pathogens and the present work concerns the Fusarium stalk rot pathogens, *Fusarium verticillioides* (Sacc.) Nirenberg (current name *Gibberella fujikuroi* (Sawada) Wollenw. var. *fujikuroi*, to be referred to as *F. verticillioides*) and *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg and the charcoal rot pathogen, *Macrophomina phaseolina* (Tassi) Goid. Fusarium stalk rot is caused by several *Fusarium* species that result in deterioration of stalk pith cells, associated with senescence during grain development (Reed and Partridge 1983). The charcoal rot fungus, *M. phaseolina*, causes infections that result in similar deterioration of the stalk, but also form dark sclerotia along the degraded vascular bundles of the colonized stalk and roots (Russin et al. 1995; Rao et al. 1980), and therefore was given the name "charcoal rot." Stalk rots are the most damaging diseases to sorghum production with incidence up to 100% in some fields (Jardine 2006) which can lead to lodging and significant biomass losses due to harvesting difficulties (Rajewski and Francis 1991). Charcoal rot and Fusarium stalk rot diseases are likely induced by environmental stresses, especially drought or heat (Tenkouano et al. 1993; Tesso et al. 2005).

It was previously proposed that the resistance or lack of increased susceptibility in *bmr6* and *bmr12* lines to

inoculations with *F. thapsinum* was due to the increase in phenolic intermediates from lignin biosynthesis previously observed that may inhibit growth within the stalk (Sattler et al. 2010b; Palmer et al. 2008). Therefore, *F. verticillioides, F. proliferatum, F. thapsinum* and *M. phaseolina* had been grown on agar media amended with varying concentrations of ferulic, sinapic, syringic, vanillic or caffeic acids (Funnell-Harris et al. 2014). *Fusarium verticillioides, F. proliferatum* and *M. phaseolina* were all inhibited at the lowest concentration of ferulic acid tested; this level was similar to the level extracted from *bmr6* plants (Palmer et al. 2008). Additionally, *F. verticillioides* was inhibited at a relatively low concentration of sinapic acid and *F. proliferatum* was inhibited at low concentrations of vanillic, sinapic and syringic acids. On the other hand, *F. thapsinum* was not inhibited at the lower concentrations of ferulic acid, but was inhibited by the lowest concentration of caffeic acid tested (Funnell-Harris et al. 2014). These results indicated that several phenolic compounds that accumulate in *bmr* as compared to wild-type plants are likely involved in inhibiting stalk rot colonization. The current research sought to determine whether *F. thapsinum* is inhibited by phenolic compounds from *bmr6* stalks.

The first goal of the current research was to assess the response of *bmr6* and *bmr12* single mutant lines and the *bmr6 bmr12* double mutant lines in two plant genetic backgrounds to the sorghum pathogens *F. verticillioides*, *F. proliferatum* and *M. phaseolina*, because responses to peduncle and/or basal stalk inoculations have not been previously reported against these commonly-encountered pathogens. The second goal was to determine whether extracts from *bmr6* plants were able to inhibit growth of the virulent pathogen, *F. thapsinum*. Thus, the results of this research provided evidence to support or refute the following hypotheses: i) *bmr6, bmr12* and double mutant lines are not more susceptible to sorghum stalk rot pathogens; and, ii) methanol extracts from *bmr* stalks are able to inhibit growth of *F. thapsinum* in vitro.

Materials and methods

Plant lines and growth conditions

Wild-type, *bmr6* and *bmr12* single mutant, and *bmr6 bmr12* double-mutant near-isogenic plants in the genetic backgrounds 'Wheatland', 'Redlan' and RTx430 (Pedersen et al. 2006; Pedersen et al. 2008) were grown from greenhouse-produced grain under greenhouse conditions. For assays, grain was sown in 25.4-cm-diameter pots containing pasteurized soil mix (one part sand, one part coarse vermiculite, one part top soil, and two parts shredded peat moss). Seedlings were culled to one plant per pot. Plants were watered throughout each experiment as needed.

Fungal isolates, culturing and maintenance

Fusarium isolates, *F. proliferatum* (H02-833S-3_PCNB, H03-555S-1_PCNB, M03-11273S-2_PCNB and M03-11335S-1_PCNB), and *F. thapsinum* (H03-11S-9) were identified using colony, conidia and conidiophore morphology (Funnell-Harris et al. 2013) and sequencing of the 5′ region of the translation elongation factor 1-α gene (Geiser et al. 2004) for previous studies (Funnell-Harris and Pedersen 2011; Funnell-Harris et al. 2010) (unpublished results) *Macrophomina phaseolina* isolates (MP01–001 and MP08-McV), kind gifts from G. Odvody (Texas A & M AgriLife Research and Extension Center, Corpus Christi, TX, USA), were hyphal-tip purified prior to use. *Fusarium verticillioides* isolate M-1141 was purchased from the Fusarium Research Center, The Pennsylvania State University, University Park, PA, USA.

Medium for maintenance of working stocks of fungi was one-half strength potato dextrose agar (PDA), prepared using potato dextrose broth (Becton Dickinson and Co. Sparks, MD, USA) and amended with 100 μmol/L ampicillin (Sigma-Aldrich, St. Louis, MO, USA). To prepare inoculum, an agar disk (5 mm in diameter) from the growing edge of 4-day-old cultures on PDA were inoculated into sterile full strength potato dextrose broth (PDB) with sterile toothpicks, previously treated to remove toxins and other inhibitors of fungal growth (Jardine and Leslie 1992; Funnell-Harris et al. 2010; Funnell-Harris et al. 2014). The broth-and-toothpick cultures were incubated for 2 weeks at 25 °C before inoculations.

Medium used for assessing growth of *F. thapsinum* (H03-11S-9) on plant phenolic extracts was peptone-glucose agar (PGA), which is 1% glucose, 0.5% peptone, 7 mM potassium phosphate monobasic, 2 mM magnesium sulfate heptahydrate, and 2.2% agar per liter. Inoculum was prepared by spreading conidia in sterile water on the surface of PGA

medium lacking amendments, 48 to 60 h prior to inoculations of test media.

Peduncle inoculations

Screens with *F. proliferatum* and *M. phaseolina* isolates were performed on cultivar RTx430. Virulence assays with *F. proliferatum* (isolate M03-11273S-2_PCNB) were conducted on near-isogenic *bmr6, bmr12*, and *bmr6 bmr12* double mutant lines, in plant genetic backgrounds RTx430 and Wheatland. Virulence assays with *M. phaseolina* isolate MP01–001 were conducted on near isogenic *bmr6, bmr12* and wild-type lines in plant genetic backgrounds Redlan and RTx430. The Redlan background was chosen because a previous study indicated cv. Redlan was highly susceptible to stalk inoculations with *M. phaseolina* (Tesso et al. 2005).

Peduncles of plants at anthesis (defined as approximately half the anthers exerted) were gently probed with a surface-disinfested awl to from a shallow hole (2 mm in diameter), then a fungal-inoculated toothpick was inserted into the hole. Eighteen days after inoculation (dai), the peduncle was split longitudinally and the length of the red to purple discoloration (the lesion) was measured. Control inoculations with sterile broth were used to account for pigmentation commonly resulting from wounding in sorghum. For isolate screens, *F. thapsinum* also was included as the high virulence control to indicate greenhouse conditions conducive to stalk rot. In general, when mean lesion length produced by a pathogen on a given line was not significantly greater than the wound response (broth control) resulting on that line, the interaction was considered to be low virulence or not pathogenic (Funnell-Harris et al. 2010).

The experimental design for the isolate screens was a randomized complete block (RCB) with six replicated blocks, one plant line, and four (*M. phaseolina*) or six (*F. proliferatum*) inoculum treatments. The experimental design for the *F. proliferatum* virulence assays on *bmr* lines was also RCB, with six replicated blocks, eight plant lines (*bmr6, bmr12, bmr6 bmr12* double mutant lines and wild-type, each in backgrounds RTx430 and Wheatland) with two inoculum treatments (*F. proliferatum* isolate M03-11273S-2_PCNB and PDB) for three repetitions. The experimental design for the *M. phaseolina* virulence assays on *bmr* plants was RCB, with six plant lines (*bmr6, bmr12* and wild-type, each in backgrounds RTx430 and Redlan) and two inoculum treatments (*M. phaseolina* isolate MP01–001 and PDB) with a total of three repetitions. The data were analyzed using PROC MIXED procedure of SAS/STAT software (SAS 2013). Data sets were analyzed for Levene's homogeneity of variance and appropriate adjustments were incorporated using the REPEATED/GROUP option. Least squares means (LSM) and standard errors (SE) are reported.

Results following inoculations with *F. proliferatum* and broth control were also analyzed by conducting *F*-tests of effect slices using the SLICE = fixed-effect option. This allowed simple comparison of the two inoculum treatments within the *bmr* genotype (SAS 2013).

Basal stalk inoculations

Response of *bmr* lines to pathogens inoculated at the base of the stalk was assessed for *F. verticillioides* (M-1141), *F. proliferatum* (M03-11273S-2_PCNB) and *M. phaseolina* (MP01–001) inoculations using a previously established protocol (Funnell-Harris et al. 2014). The two *Fusarium* spp. were inoculated onto *bmr6, bmr12* and *bmr6 bmr12* double mutant and near isogenic wild-type lines in the backgrounds RTx430 and Wheatland while *M. phaseolina* was inoculated onto *bmr6, bmr12* and wild-type lines in the backgrounds Redlan and RTx430, because the double mutant was not available in Redlan, chosen because a previous study indicated this cultivar was highly susceptible to *M. phaseolina* (T. T. Tesso et al. 2005). For *F. verticillioides* and *F. proliferatum*, varying inoculation and measurement times were assessed but the previously published protocol proved to be optimal. Briefly, 10 weeks after planting, the second internode of each plant was inoculated by gently probing the stalk with a surface disinfested awl and inserting a fungal infested toothpick, or one incubated in sterile PDB, into the resulting shallow hole. Lesion length measurement was conducted at 32 dai (Funnell-Harris et al. 2014). When mean lesion length produced by a pathogen on a given line was not significantly greater than the wound response (broth control) resulting on that line, the interaction was considered as having low virulence or not pathogenic.

The experimental design for *F. verticillioides* or *F. proliferatum* inoculations was RCB, with eight blocks each with eight plant lines (*bmr6, bmr12* and *bmr6 bmr12* double mutant and wild-type lines each in the backgrounds RTx430 and Wheatland) and two

inoculum treatments (the fungus and PDB) with a total of three repetitions. For *M. phaseolina* inoculations, the experimental design was again RCB with six blocks, each with six plant lines (*bmr6, bmr12* and wild-type lines each in the backgrounds Redlan and RTx430) and two inoculation treatments (*M. phaseolina* and PDB); this was performed in a single repetition.

In vitro growth of *F. thapsinum* on extracts from *bmr6* and wild-type plants

The stalk internode between the flag leaf and the uppermost true leaf was removed from BTx623 wild-type and *bmr6* plants (approx. 30 each), after anthesis during the 2014 field season. This internode had been chosen as the phenotype was most pronounced in *bmr6* plants, as compared with wild-type plants (Dowd and Sattler 2015; Dowd et al. 2016). The stalks were stored briefly on ice then at −80 °C. The stalk tissues were lyophilized (Freezone 6, Labconco, Kansas City, MO, USA) and ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) then extracted with 100% methanol (MeOH) for 30 min. by inversion mixing at 4 °C. After centrifugation, the supernatant was removed to a fresh tube; the pellet was again extracted with 100% MeOH. The supernatant from each extraction was combined and dried under vacuum. After resuspension in MeOH, extracts were stored overnight at −80 °C. The volume of MeOH used to resuspend the pellet corresponded to the amount needed to approximate concentrations of the extracted solutes from fresh stalk tissue once incorporated into PGA media.

In order to determine residual sugars (glucose, sucrose and fructose) in MeOH extractions, three small-scale (0.05-fold) extractions were conducted, as described above, and residual sugars present in extracts were determined with three technical repetitions per extraction (Dionex ICS-3000 ion chromatography system, Sunnyvale, CA, USA). Least Squares Means (LSM) were determined and differences in LSM were performed using PROC MIXED procedure of SAS/STAT software (SAS 2013). Residual glucose, sucrose and fructose resulting from MeOH extraction of lyophized, ground tissues from *bmr6* plants were significantly greater than those from wild-type plants (Table 1). Therefore, a total of three controls were included in the experiment: PGA medium with 8% MeOH, and PGA medium with 8% MeOH and the additional glucose, sucrose and fructose for each extraction, according to Table 1. It was previously shown that 8% MeOH added to PGA did not affect growth of *F. thapsinum* (Funnell-Harris et al. 2014).

PGA with MeOH (control), sugars and MeOH (extraction method control, one for wild-type and one for *bmr6* plant tissues), or plant extracts in MeOH (wild-type or *bmr6*), was dispensed into 12-well cell culture plates (Costar, Corning Life Sciences, Corning, NY, USA), 500 μl per well. Each plate had four wells with the same treatment (across, horizontally) and two or three different treatments (vertically) were included in each plate. Treatments were randomized by plate with rows within each plate then randomized. For each assay, inoculum was prepared by spreading spore suspensions prepared in sterile purified water (Labconco, Kansas City, MO, USA) onto four 35-mm Petri dishes with1 ml PGA medium and incubated 48–60 h in a moist chamber at room temperature. To inoculate media in culture plate wells, an agar plug (one-half a 4-mm diameter disk) was placed at the edge of the agar medium, near the wall, in each well. The plates were incubated at 22 °C in a moist chamber on stationary shelves within a refrigerated incubator (Shel Lab Shaking Incubator, Cornelius, OR, USA). To measure radial growth, radius from the agar plug was scored on the bottom of each well using a scalpel. Daily, beginning at day 0 (the edge of the agar plug), growth was indicated by scoring along the radius under a dissecting microscope for seven (assay 2) or eight (assays 1 and 3) days.

For the experimental design, plates containing the four replicate treatments were completely randomized. Three repetitions were performed, separated by time and location in the incubator. Data were analyzed using PROC MIXED procedure of SAS/STAT software

Table 1 Residual glucose, sucrose and fructose (mg g^{-1}) following methanol extractions of lyophilized and ground tissues from the stalk internode between the flag leaf and the uppermost true leaf of field-grown *brown midrib-6* (*bmr6*) and wild-type sorghum plants

	bmr6	Wild-type
Glucose	21.82*[a] ± 0.39	14.82 ± 0.39
Sucrose	62.97* ± 1.28	53.23 ± 1.28
Fructose	17.70* ± 0.42	15.02 ± 0.42

[a] Least squares means (LSM) and standard errors (SE) are shown. Differences of LSM were determined using PROC MIXED procedure of SAS/STAT software (SAS 2013). The asterisks indicate that the sugar level is significantly greater at $P < 0.01$

(SAS 2013). Repetitions were considered random effects. The KENWARD-ROGER option was specified to calculate denominator degrees of freedom to reduce standard error and *F*-statistic bias (Kenward and Roger 1997; Littell et al. 2006). Medium [MeOH, S-WT (MeOH with sugars according to wild-type plant extract), S-*bmr6* (MeOH with sugars according to *bmr6* extract), MeOH extract from *bmr6* plants and MeOH extract from wild-type (WT) plants] was considered a fixed effect in the model used to analyze radial growth, and measurements across time on the same well were considered repeated measures. Data were analyzed for Levene's homogeneity of variance and appropriate adjustments were incorporated using the REPEATED/GROUP option. A linear model was used to estimate growth rate on each medium. Slopes were output using the SOLUTION option and the NOINT option was used to force the intercept to zero at the initiation of the experiment. Confidence intervals for the slopes were set at $P = 0.05$ using the CL option. Comparisons among media were made using single degree of freedom contrasts.

Results

Response of *bmr* to *F. verticillioides* basal stalk inoculations

Peduncle inoculations of *F. verticillioides* onto *bmr6* and *bmr12* plants were previously conducted, showing that *bmr* lines resulted in significantly shorter mean lesion lengths than near-isogenic wild-type plants (Funnell-Harris et al. 2010). In the present study, the base of the stalk of *bmr6, bmr12, bmr6 bmr12* double mutant and near-isogenic wild-type lines, in two backgrounds (RTx430 and Wheatland), were inoculated with *F. verticillioides* (Fig. 1a, b). In this case, there were no significant differences in mean lesion lengths between *bmr* lines as compared with wild-type. RTx430 *bmr12* mean lesion length was not significantly different than that of its control inoculation (wound response) (Fig. 1A), which suggests that *F. verticillioides* had low virulence in this interaction. In the case of cv. Wheatland background, *F. verticillioides* inoculation resulted in mean lesion lengths significantly greater than the control response on each line, but those on *bmr* lines were not significantly different from those resulting on the wild-type line (Fig. 1b).

Response of *bmr* to *F. proliferatum* peduncle and basal stalk inoculations

Peduncle inoculations with four *F. proliferatum* grain isolates were compared with the highly virulent *F. thapsinum* isolate commonly used in these assays (H03-11S-9) (Funnell-Harris et al. 2010). All mean lesion lengths of the *F. proliferatum* isolates, ranging from 63.7 to 89.7 mm, were not significantly different ($P \geq 0.26$) from that of *F. thapsinum* (mean lesion length 85.7 mm; SE = 13.9). Isolate M03-11273S-2_PCNB was chosen (mean lesion length 89.7 mm) to conduct peduncle and basal stalk inoculations to assess response of *bmr* and wild-type plants.

Peduncles of *bmr6, bmr12*, *bmr6 bmr12* double mutant and wild-type plants in two backgrounds were wound inoculated with *F. proliferatum* and the broth control, and mean lesion lengths compared. Interactions between *bmr* genotype × inoculum and line × *bmr* genotype × inoculum were not significant ($P \geq 0.07$). In background RTx430, single degree of freedom comparison tests between *F. proliferatum* inoculated plants indicated that *bmr6 bmr12* double mutant had shorter mean lesion lengths than the wild-type, while the lengths of the discoloration in broth inoculated control plants were similar (Fig. 2a). In the Wheatland background, the mean lesion lengths produced on *bmr6* and *bmr6 bmr12* double mutant plants were not significantly different from the wound response resulting from control inoculations on these lines (Fig. 2b), which suggested *F. proliferatum* had low virulence on these lines. However, the mean lesion length of the control inoculation on wild-type Wheatland plants was significantly greater than those on the *bmr* lines. Therefore, partitioned analyses (tests of simple effects) of inoculum on the line × *bmr* genotype interactions were conducted. This analysis indicated that the mean lesion lengths resulting from inoculation with *F. proliferatum* on RTx430 *bmr6, bmr12* and *bmr6 bmr12* double mutant and wild-type lines were significantly different from the control treatment on the same line ($P \leq 0.01$). In background Wheatland, mean lesion lengths following *F. proliferatum* inoculations on *bmr12* and wild-type lines were significantly different than discolorations resulting from control inoculations ($P \leq 0.01$), while mean lesion lengths resulting from *F. proliferatum* and

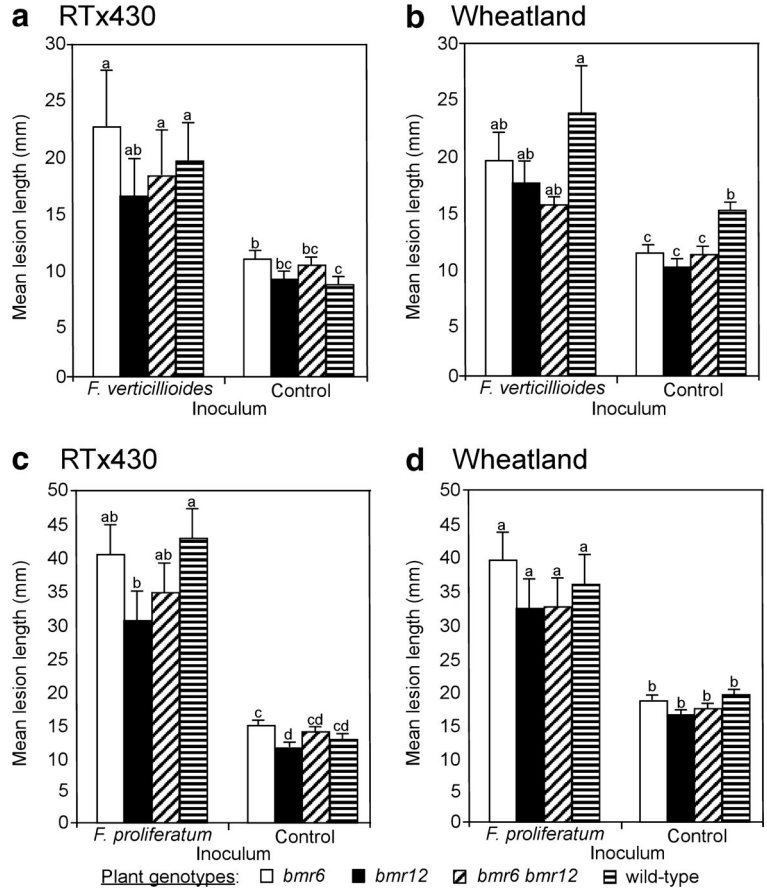

Fig. 1 Response of near isogenic *brown midrib* (*bmr*)-*6*, *bmr12*, *bmr6 bmr12*, and wild-type lines in the sorghum genetic backgrounds **a**., **c**. RTx430; and **b**., **d**. Wheatland, to basal stalk (second internode) inoculations with pathogens, **a**., **b**. *Fusarium verticillioides*; and **c**., **d**. *Fusarium proliferatum*. Plants were inoculated with toothpicks incubated with *Fusarium* species or broth (control). Control inoculations were included for each to indicate wound response of the plant. Lengths of discolorations resulting on split stalks were measured. Mean lesion lengths and positive standard errors are shown. *Bars* indicated with the same letter are not significantly different

control inoculations on *bmr6* and *bmr6 bmr12* double mutant lines were not ($P \geq 0.10$). This analysis confirmed that *F. proliferatum* inoculations of Wheatland *bmr6* and *bmr6 bmr12* double mutant lines yielded similar responses as the broth control. Therefore, these analyses indicated that *F. proliferatum* has low virulence on *bmr6* and *bmr6 bmr12* double mutant lines.

Basal stalk inoculations with *F. proliferatum* also were performed on *bmr6, bmr12* and *bmr6 bmr12* double mutant plants in the same two plants backgrounds. Interactions *bmr* genotype × inoculum and line × *bmr* genotype × inoculum were not significant ($P \geq 0.25$). In RTx430, the mean lesion lengths resulting on *bmr6* and *bmr6 bmr12* double mutant plants were not significantly different than those resulting on wild-type plants (Fig. 1c). The mean lesion length resulting on *bmr12* plants inoculated with *F. proliferatum* was significantly less than those on wild-type plants. In background Wheatland (Fig. 1d), mean lesion lengths on all three *bmr* lines were not significantly different from that resulting on wild-type plants, following inoculation with *F. proliferatum*.

Response of *bmr* plants to *M. phaseolina* peduncle inoculations

Two *M. phaseolina* isolates were screened for virulence using peduncle inoculations, as compared with *F. thapsinum*. Although *M. phaseolina* isolate MP01–001 resulted in significantly shorter mean lesion lengths (39.8 mm) than *F. thapsinum* (mean lesion length 64.8 mm; SE 7.7; $P = 0.02$), it also had mean lesion lengths greater than MP08-McV (15.7 mm; $P = 0.02$) and the broth control (7.8 mm; $P < 0.01$). Therefore, isolate MP01–001 was chosen to assess response of *bmr* lines to *M. phaseolina*.

Peduncle inoculations were conducted on near-isogenic *bmr6*, *bmr12* and wild-type lines in the backgrounds Redlan and RTx430, both of which had

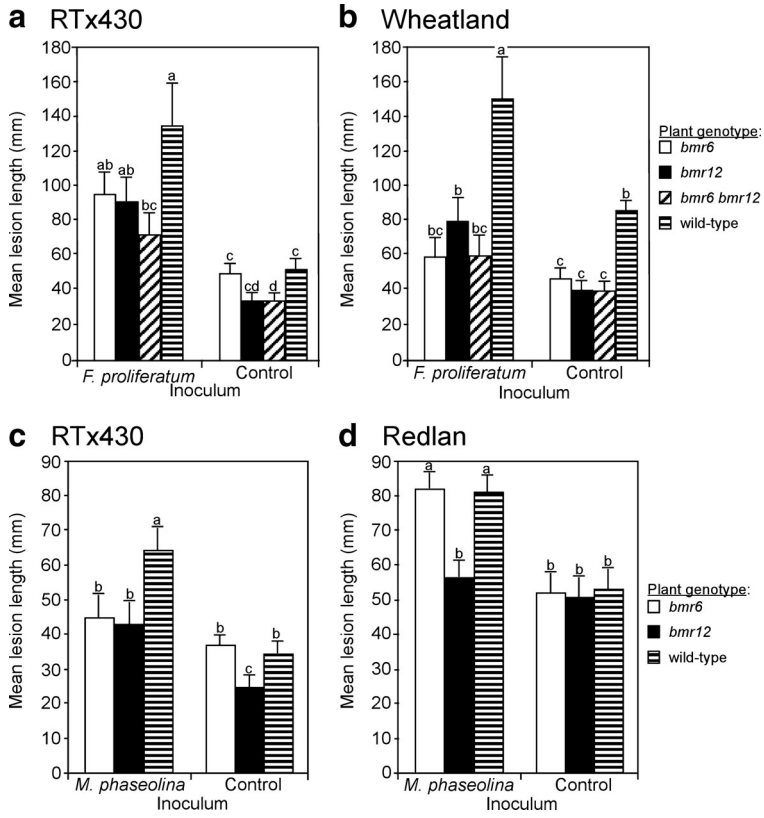

Fig. 2 Response of near isogenic *brown midrib* (*bmr*) lines, as compared with wild-type, to peduncle (top of the stalk) inoculations with stalk fungi **a**., **b**. *Fusarium proliferatum*; and **c**., **d**. *Macrophomina phaseolina*. Near isogenic lines *bmr6, bmr12, bmr6 bmr12* and wild-type lines in the genetic backgrounds **a**. RTx430 and **b**. Wheatland were inoculated with *F. proliferatum* or control, while *bmr6, bmr12* and wild-type lines in the backgrounds **c**. RTx430 and **d**. Redlan were inoculated with *M. phaseolina* and control. Plants were inoculated with toothpicks incubated with fungus or broth (control). Control inoculations were included for each to indicate wound response of the plant. Lengths of discolorations resulting on split peduncles were measured. Mean lesion lengths and positive standard errors are shown. Bars indicated with the same letter are not significantly different

previously been reported to be susceptible to *M. phaseolina* (Funnell-Harris et al. 2016; T. T. Tesso et al. 2005). Resulting mean lesion lengths on *bmr6* (RTx430) and *bmr12* (Redlan and RTx430) were significantly less than on corresponding wild-type lines (Fig. 2). For control inoculations, there were no significant differences between *bmr* lines and control in the cv. Redlan background (Fig. 2a). However, *bmr12* had a reduced wound response in comparison to the other genotypes in RTx430 background (Fig. 2b).

Basal stalk inoculations, using the same procedure as used for *F. verticillioides* and *F. proliferatum*, were also conducted with *M. phaseolina* as the inoculum, along with the broth control. However, this protocol yielded no significant differences in mean lesion lengths due to inoculum ($P = 0.21$).

In vitro growth of *F. thapsinum* on phenolic extracts from *bmr6* and wild-type plants.

Fusarium thapsinum was grown on media amended with methanol plant extracts from wild-type and *bmr6* plants added to PGA medium, PGA media with the addition of methanol and sugars at the concentration present in the extracts and PGA media with added methanol. The fungus grew significantly faster on media containing plant extracts than PGA media or the two sugar amended versions (Table 2). The fungus also grew significantly faster on medium with *bmr6* extracts than the one with wild-type extracts. The greater amounts of glucose, sucrose and fructose were found in *bmr6* extracts, and when these sugar concentrations were added to the medium along with methanol (S-*bmr6*), growth was increased over the control with sugar concentrations found in wild-type extracts (S-WT) (Table 2).

Discussion

This research provides strong evidence to further support the first hypothesis: *bmr6, bmr12* and double mutant lines are not more susceptible than wild-type to sorghum stalk rot pathogens. It was demonstrated that near-isogenic *bmr6, bmr12* and *bmr6 bmr12* double mutant lines in two backgrounds are not more susceptible to the Fusarium stalk rot pathogens *F. verticillioides*

Table 2 Slopes (mm of growth per day) resulting from regression analysis of radial growth of *Fusarium thapsinum* on peptone glucose agar amended with methanol extracts from *brown midrib-6* (*bmr6*) or wild-type (WT) plants and control media amended with methanol and additional sugars (S-*bmr6* or S-WT) or methanol (MeOH)

Medium[y]	Slope (mm day^{-1})[z]
bmr6	1.78a ± 0.04
WT	1.56b ± 0.05
S-*bmr6*	1.23c ± 0.04
S-WT	1.06d ± 0.04
MeOH	1.00d ± 0.04

[y] The stalk internode between the flag leaf and the uppermost true leaf was removed from wild-type and *bmr6* plants (approx. 30 each), grown during the 2014 field season. Tissues were dried and ground then extracted with 100% methanol. Extracts were added to medium at 8% (v/v) so that extract concentrations were similar to those in plants. For sugar (S) control media, glucose, sucrose and fructose were added to media according to results from small-scale methanol extractions (see Table 1)

[z] Slopes calculated from estimates for fixed effects. Slopes with different letters are significantly different at $P < 0.05$

and *F. proliferatum*. It was previously shown that several *bmr* lines were not more susceptible to stalk inoculations by *F. thapsinum*, and that *bmr6* and *bmr12* lines could exhibit resistance to peduncle inoculations by *F. thapsinum* and *F. verticillioides* (Funnell-Harris et al. 2010; Funnell-Harris et al. 2014). In the current study, inoculations with the highly virulent pathogen *F. proliferatum* demonstrated that *bmr* lines had increased resistance to basal stalk inoculations of RTx430-*bmr12* and to peduncle inoculations of RTx430-*bmr6 bmr12*, Wheatland-*bmr6* and Wheatland-*bmr6 bmr12*, as indicated by significantly smaller mean lesion lengths than those resulting on wild-type (Figs. 1,2). Although *bmr* mutations can impart resistance responses to some pathogens, as previously observed, genetic background (Funnell and Pedersen 2006; Funnell-Harris et al. 2014), environment (Funnell-Harris et al. 2010; Funnell-Harris et al. 2016) and inoculation site (present work) all influence this response. Nonetheless, across both genetic backgrounds, mean lesion lengths resulting on *bmr12* (31.6 ± 2.7 mm) lines resulting from basal stalk inoculations with *F. proliferatum* were significantly less than those on wild-type (40.2 ± 2.7 mm) ($P = 0.04$). This study also demonstrated that the near-isogenic *bmr6* and *bmr12* lines were not more susceptible to peduncle inoculations with the charcoal rot pathogen, *M. phaseolina* (Fig. 2). But excitingly, this study demonstrated that *bmr6* (RTx430) and *bmr12* (Redlan and RTx430) lines exhibited increased resistance to this destructive pathogen (Fig. 2c, d). This result is consistent with observations from inoculations of several different *bmr* lines at the base of field-grown plants, which included *bmr6* or *bmr12* mutants, using a technique similar to that described in the present work: the mutations did not affect disease severity when compared with the wild-type (T. Tesso and Ejeta 2011).

One possible explanation for increased resistance (or reduced susceptibility) of *bmr* lines to *Fusarium* stalk pathogens observed in this and previous studies is the changes in levels of phenolic compounds (Palmer et al. 2008) that have been shown to be inhibitory to these fungi (Beekrum et al. 2003; Ferrochio et al. 2013; Funnell-Harris et al. 2014; Samapundo et al. 2007). In recent studies, it was demonstrated that field-grown leaves of *bmr6* plants could sustain less damage when fed to insect caterpillars (European corn borers [*Ostrinia nubilalis* (Hübner) (Lepidopera: Pyralidae)], corn earworms [*Helicoverpa zea* (Bodie) (Lepidopera: Noctuidae)] and fall armyworms [*Spodoptera frugiperda* (J.E. Smith) (Lopidopera: Noctuidae)]) but environment could affect the response (Dowd et al. 2016; Dowd and Sattler 2015). However, when corn earworms or fall armyworms were fed the uppermost stalk internode from plants from the same three environments, significantly greater mortality was observed when fed *bmr6* plants, as compared with wild-type (Dowd et al. 2016). Thus, *bmr6* plants had greater toxicity toward these pests than wild-type plants and it appeared to be consistent across environments.

It was previously demonstrated that total soluble aromatics were significantly increased in *bmr6* lines as compared with near-isogenic wild-type lines (Palmer et al. 2008). One of the phenolic intermediates significantly increased in these lines was ferulic acid, which had been shown to inhibit in vitro growth of *F. verticilioides*, *F. proliferatum* and *M. phaseolina* at biologically relevant levels (Funnell-Harris et al. 2014). Ferulic acids and their derivatives have been implicated in plant resistance to fungal pathogens (Chandrashekar and Satyanarayana 2006; Lanoue et al. 2010; Picot et al. 2013; Sampietro et al. 2013), pathogenic nematodes (Valette et al. 1998; Wuyts et al. 2007) and insect pests (Dhillon and Chaudhary 2015; Garcia-Lara et al. 2010; Mao et al. 2007; Santiago et al. 2006). However, *F. thapsinum*, a sorghum stalk pathogen of world-wide

distribution (Bramel-Cox and Claflin 1989; Frowd 1980; Petrovic et al. 2009), was shown to be tolerant to biologically relevant levels of ferulic acid (Funnell-Harris et al. 2014). Therefore, *F. thapsinum* was chosen in the current study to be assessed for in vitro growth on soluble phenolic extractions from *bmr6* and wild-type plants to determine whether there are components of soluble phenolic metabolites from *bmr6* plants inhibitory to this pathogen. The results of this study did not support the second hypothesis: soluble phenolic stalk extracts from *bmr* plants are able to inhibit growth of *F. thapsinum* in vitro. The surprising result was that *F. thapsinum* grew at a significantly greater rate on the *bmr6* extracts than on wild-type extracts or the controls. The methanol extractions removed some glucose, sucrose and fructose from the stalk tissue and significantly greater amounts of these sugars were present in the *bmr6* extracts (Table 1); this difference may have contributed to the increased growth rate of the *F. thapsinum* isolate on *bmr6* extracts (Table 2). Nonetheless, there was no evidence of inhibitory factors present at levels adequate to reduce growth of *F. thapsinum*, in the presence of these increased sugars. Previous research with *bmr* maize and sorghum suggested that there was no clear association between total sugars and *bmr* (Fritz et al. 1990; Hanna et al. 1981; Marita et al. 2003), but a more recent study showed that *bmr12* hybrids had moderately increased sugars in the biomass compared with wild-type (Sattler et al. 2010b). Taken together, these results lead to the conclusion that there are other factors involved in the observed resistance of *bmr* plants to *F. thapsinum* in addition to changes in levels of free phenolic metabolites from the lignin biosynthesis pathway. For example, the inhibitory factor may be cell wall bound phenolic compounds (Mandal et al. 2009; Palmer et al. 2008; Santiago et al. 2007), which are covalently linked to cell wall components and are not extractable in methanol. Another possibility may be differences in inducible defense compounds between *bmr6* and wild-type (Afroz et al. 2011; Christensen and Kolomiets 2011; Gozzo and Faoro 2013). For example, one of the pathways for biosynthesis of the signaling molecule salicylic acid involves cinnamic acid, a component of the monolignol biosynthesis pathway (Humphreys and Chapple 2002; Lo and Nicholson 1998; Metraux 2002). An increase in levels of phenylpropanoid pathway intermediates in *bmr6* may increase salicylic acid levels in response to pathogens or other stresses resulting in greater or more rapid induction of defense compounds (Funnell-Harris et al. 2010). It is unlikely that increased levels of inducible defense compounds would be present at significant levels in the extracts from the current study, because stalk extracts came from apparently healthy field grown plants. Multiple defense components, such as a combination of inducible compounds as well as cell wall bound phenolics, could also be at play in the increased resistance to stalk pathogens observed in *bmr* plants.

In summary, this study demonstrated that the *bmr* lines, *bmr6, bmr12* and *bmr6 bmr12* double mutant are not more susceptible to the Fusarium stalk rot pathogens, *F. verticillioides, F. proliferatum* and *bmr6* and *bmr12* lines are not more susceptible to the charcoal rot pathogen, *M. phaseolina*, and that some *bmr* lines exhibited increased resistance to the highly virulent pathogen Fusarium stalk rot pathogen, *F. proliferatum* and the destructive charcoal rot pathogen, *M. phaseolina*. In a previous study, these three pathogens were inhibited in vitro by the phenolic metabolite ferulic acid, which had been found at increased levels in soluble phenolic extracts from *bmr6* plants (Funnell-Harris et al. 2014; Palmer et al. 2008). However, another highly virulent pathogen, *F. thapsinum*, which had been shown to result in significantly smaller lesions on *bmr6* plants compared with wild-type (Funnell and Pedersen 2006; Funnell-Harris et al. 2010), was not inhibited by ferulic acid at low levels (Funnell-Harris et al. 2014). Therefore, response of this pathogen to stalk extracts was assessed in the current study. The results of this study suggested that multiple factors are involved in resistance to different stalk pathogens in *bmr* lines and some may be cell wall bound or inducible defenses.

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